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13. ABSTRACT (Maximum 200 Words)

We have changed from using rats to mice. This change was approved by the DOD review committee. Or hypothesis is that if we render the host immune cells insensitive to TGF-β, these immune cells would be able to eradicate tumor cells. There are three tasks. Task 1 was to establish a dominant negative TGF-β type II receptor bearing bone marrow cells, using GFP as a selectable marker. Task 2 was to test the role of TGF-β insensitivity in immune cell development and differentiation. Task 3 was to assess the anti-tumor activity in host animals bearing TGF-β insensitive bone marrow cells. A retro viral based expression construct containing a dominant negative type II receptor for TGF-β and GFP was constructed and tested for expression efficacy. This construct was infected in bone marrow cells of C57BL/6 mice ex vivo. Following infection, bone marrow cells were transplanted into C57BL/6 recipients, which received prior total body irradiation. These recipients survived the transplant and also eliminated tumor cells injected via intra-venous route. The proposed study is highly relevant to prostate cancer research. In this study, we have demonstrated that TGF-β insensitive bone marrow cells transplanted to recipient hosts are able to eliminate cancer cells.

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Mouse prostate cancer, gene therapy, TGF-β, TGF-β receptor

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Huang X, Lee C. (2003) From TGF-β to Cancer Therapy. Current Drug Targets 4:243-250.

Introduction

Our objective is to develop a novel gene therapy program for cancer. This proposal explored the possibility of using TGF-β insensitive bone marrow cells in the development of an anti-cancer therapeutic strategy in a mouse prostate cancer system. We used the TRAMP prostate cancer model in C57BL/6 mice. We have developed a system in which we are able to introduce the type II TGF-β receptor dominant negative gene (TβRIIDN) into mouse bone cells, rendering them insensitive to TGF-β. Our studies showed that, if bone cells were rendered insensitive to TGF-β, they were able to inhibit tumor growth, following bone marrow transplant into recipient syngeneic hosts. These observations are significant with regard to our efforts to cure cancer and they have provided a possible novel approach to combat cancer.

Body

Progress:

Major changes in the original proposal:

The study was originally proposed to use the MATALyLu tumor system in rats. We have changed to the use of the TRAMP-C2 tumor system in mice. Aside from changes from MATALyLu in rats to TRAMP-C2 in mice, all proposed procedures and overall strategies remain the same. The major reason for this change is that most commercial antibody reagents are available in mice.

Task 1. **To establish an in vivo model system to study the feasibility of bone marrow cells bearing dominant negative TβRII (months 1-6).**

Work accomplished: We have completed all proposed studies in this task. A manuscript was published reporting the findings of this experiment (Shah et al, 2002a). Briefly, as indicated in our original proposal, we inserted a dominant negative type II TGF-β receptor into MSCV retrovirus gene, which also expressed green fluorescent protein (GFP) as a marker. The control vector contained the GFP only. Bone marrow cells of the donor animals were infected with this virus. The infection rate was greater than 95%. Recipient animals were subjected to total body irradiation and then transplanted with the viral particles via the tail vein. Animals survived for more than three months are indicative of successful engraftment of the bone marrow cells. Figure 1 illustrates the overall procedure of this experiment.

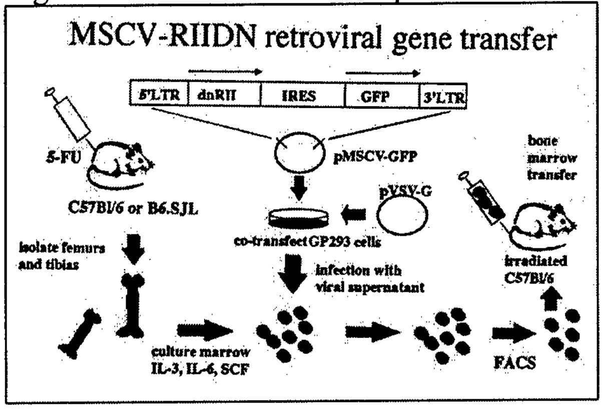

Figure 1. Bone Marrow Transplantation Protocol. Bone marrow cells isolated from the 5FU-treated donor mice are infected with TβRIIDN retrovirus packaged using GP293 cell line and transplanted into the lethally-irradiated recipient mice through tail vein injection.

Task 2: **Role of TGF-β insensitivity in immune cell development and differentiation.**

Work accomplished: We have completed all proposed studies in this task. Animals received TGF-β insensitive bone marrow transplants survived the engraftment. However, they eventually developed widespread inflammatory disease, affecting internal organs such as the lung, liver, kidney, etc. at 3-4 months following transplant. Recipient animals died at 7 months post-transplant (Figure 2). Inspection of splenic cells revealed an interesting phenomenon. Reports in the literature indicated that TGF-β knockout transgenic animals displayed an expansion of T cells. However, in the present study, T cell expansion was not observed in these recipient mice. Instead, there was an expansion of myeloid cells (Figure 3A). However, most of the T cells were differentiated to memory phenotype (Fig. 3B). These results indicated that TGF-β acts as a negative regulator of the immune system, and the lack of TGF-β signaling leads to proliferation in myeloid cells and inflammatory diseases. These observations were published in the Journal of Immunology (Shah et al, 2002a).

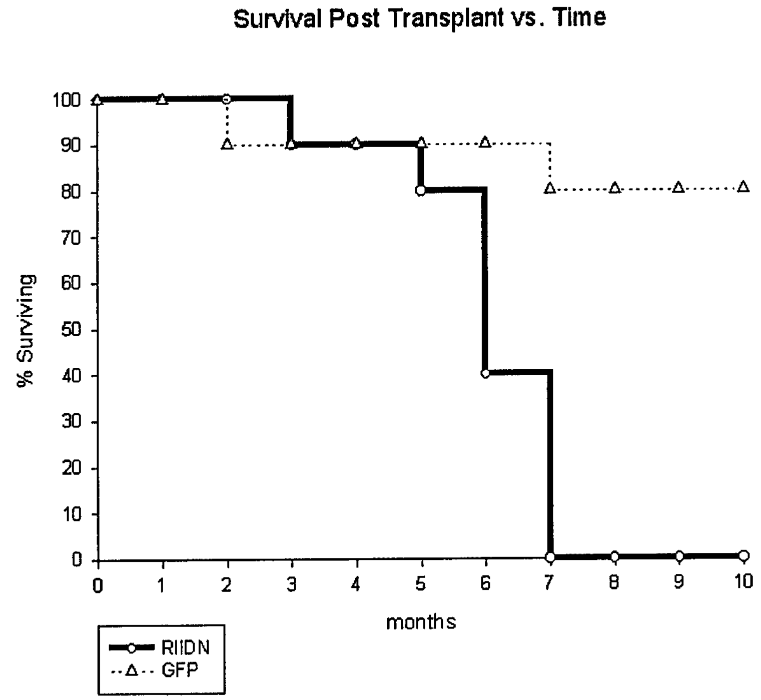

Figure 2: Survival time course of C57BL/6 mice received transplant of TGF-β insensitive bone marrow cells.

Figure 3A: **Figure 3B:**

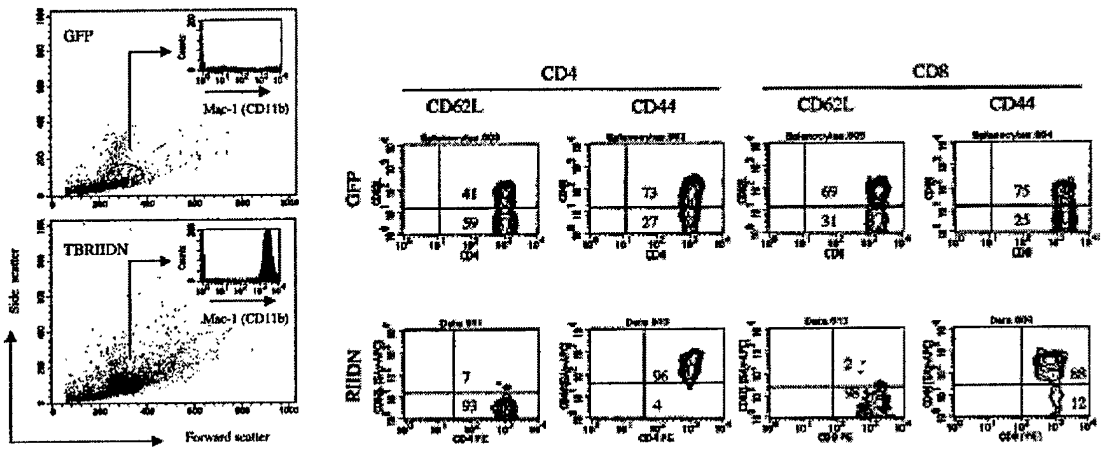

Figure 3: Expansion of Mac-1^+ cells in splenocytes (A) and T cell phenotype analysis (B) of TβRIIDN reconstituted mice vs. GFP control.

Task 3: The anti-tumor activity in host animals bearing TGF-β insensitive bone marrow cells.

Work accomplished: We have completed the task of xenograft growth of the TRAMP-C2 mouse prostate cancer cells in host animals bearing TGF-β insensitive bone marrow cells. In order to determine the efficacy of the TGF-β insensitive bone marrow treatment on metastatic tumor formation in a model of prostate cancer, we subsequently challenged male C57BL/6 mice with intravenous administration of $5x10^5$ TRAMP-C2 cells, and monitored the mice. At 3 weeks post challenge, macroscopic tumor formation was difficult to detect in either the treated or untreated controls. Further examination of histological specimens of mice sacrificed at 21 days post tumor challenge, micrometastatic lesions were already visible in the GFP group but not in the TβRIIDN group (data not shown). A second group of mice was tumor-challenged and monitored for period of

8 weeks, by which point the survival of the wild type and GFP control mice was 0% (0/5 each group by week 7, Figure 3a), while the survival of the TβRIIDN-BM treated cohort was 100% (5/5). By week 9, one animal in the TβRIIDN-BM group died, leaving the overall survival rate of 80% (4/5) for this group. Results of statistical analysis, using the log ranking test, indicated p<0.05 between the TβRIIDM-BM and the other two control groups. Post mortem analysis of the untreated or vector-control treated animals indicated a significant tumor burden evident in the lung tissue of each mouse (Figure 4), while the lungs of TβRIIDN mice remained metastases free. From this data, we conclude that targeting immune TGF-β signaling with bone-marrow directed retroviral therapy is an effective means of preventing metastatic prostate tumor growth in mice. The above results were published in Cancer Research (Shah et al, 2002b)

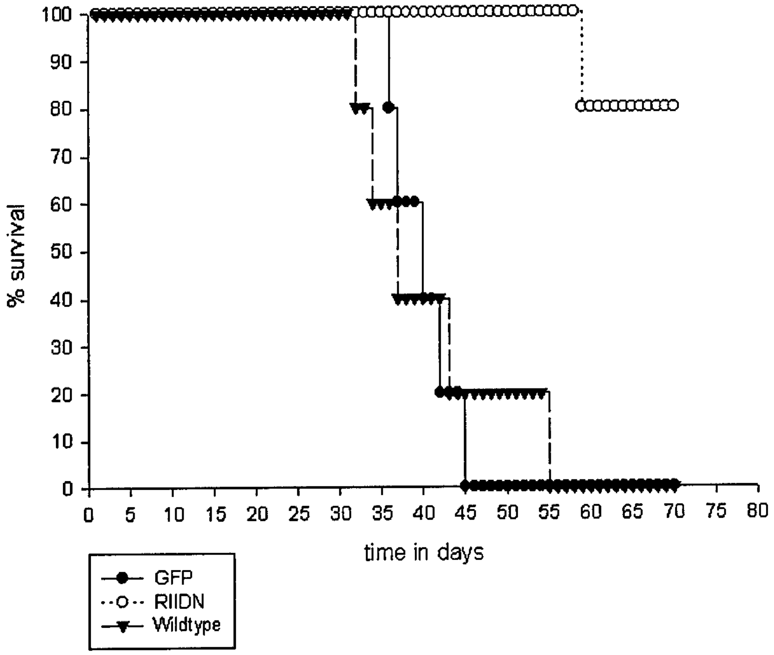

Figure 4: Kaplan-Meier survival curve of C57BL/6 mice challenged with 5 x10^5 TRAMP-C2 cells via tail vein injection after transplantation with 2–4 x 10^6 syngeneic BM cells transduced with TβRIIDN-expressing retrovirus, GFP control virus, or uninfected wild-type BM cells.

Key Research Accomplishments:

1. We have made a retroviral constructs that expresses the dominant negative type II receptor for transforming growth factor-beta (TGF-β).
2. We have successfully infected mouse bone marrow cells with the above viral construct so that these bone marrow cells are insensitive to TGF-β.
3. When these TGF-β insensitive bone marrow cells are transplanted to recipient mice, they were able to engraft and generate immune cells of various lineages that are all insensitive to TGF-β.
4. Finally, recipient animals, transplanted with TGF-β insensitive bone marrow cells, were able to eliminate tumor cells; while animals received the wild type bone marrow cells were unable to eradicate cancer cells.
5. A side effect of this approach is that recipient animals eventually developed widespread inflammatory disease, causing eventual death in the hosts.
6. Our future studies will focus on developing a TGF-β insensitive immune cells that do not cause the development of widespread inflammatory disease in the hosts.

Reportable Outcomes:

Shah AH, Tabayoyong WB, Kim SJ, van Parijs L, Kimm S, Lee C. (2002a) Reconstitution of lethally irradiated mice with TGF-β insensitive bone marrow leads to myeloid expansion and inflammatory disease. Journal of Immunology 169:3485-3491.

Shah AH, Tabayoyong WB, Kundu SD, Kim SJ, van Paris L, Liu VC, Kwon E, Greenberg NM, Lee C. (2002b) Suppression of tumor metastasis by blockade of TGF-β signaling in bone marrow cells through a retroviral mediated gene therapy in mice. Cancer Research 62:7135-7138.

Huang X, Lee C. (2003) From TGF-β to Cancer Therapy. Current Drug Targets 4:243-250.

The most exciting outcome of this research is that mankind is one step closer to curing cancer. The present study represents a pre-clinical research, using the mouse prostate cancer as the model system. Our findings clear demonstrated that the present approach will be effective in eradicating cancer in syngeneic hosts. We still have some obstacles. For example, host animals in the present study developed widespread inflammatory disease, which causes eventual death in the host animals. Our future effort will focus on modifying the present strategy so that cancer cells can be eliminated without the development of the undesirable side effect of widespread inflammatory disease.

- Patents: None
- Clinical translational research: None
- Employment, promotion, or career development: None
- Other relevant items: None

Conclusion:

These results have led us to conclude that it is possible for us to develop a similar anti-cancer program for human prostate cancer patients. The over-production of TGF-β in prostate cancer cells on tumor progression was the subject of our Phase I Study. Our findings in the Phase II study led us to conclude that TGF-β insensitive immune cells can be used as a possible anti-tumor strategy (Huang and Lee, 2003). Our future effort will focus on the development of a strategy whereby TGF-β insensitive immune cells can be modified in that we will be able to eradicate tumor cells but avoid the development of widespread inflammatory disease in the host.

References:

Shah AH, Tabayoyong WB, Kim SJ, van Parijs L, Kimm S, Lee C. (2002a) Reconstitution of lethally irradiated mice with TGF-β insensitive bone marrow leads to myeloid expansion and inflammatory disease. Journal of Immunology 169:3485-3491.

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Reconstitution of Lethally Irradiated Adult Mice with Dominant Negative TGF-β Type II Receptor-Transduced Bone Marrow Leads to Myeloid Expansion and Inflammatory Disease[1]

Ali H. Shah,* William B. Tabayoyong,* Simon Y. Kimm,* Seong-Jin Kim,† Luk van Parijs,‡ and Chung Lee[2]*

TGF-β regulation of immune homeostasis has been investigated in the context of cytokine knockout (TGF-β null) mice, in which particular TGF-β isoforms are disrupted throughout the entire organism, as well as in B and T cell-specific transgenic models, but to date the immunoregulatory effects of TGF-β have not been addressed in the context of an in vivo mouse model in which multi-isoform TGF-β signaling is abrogated in multiple leukocyte lineages while leaving nonhemopoietic tissue unaffected. Here we report the development of a murine model of TGF-β insensitivity limited to the hemopoietic tissue of adult wild-type C57BL/6 mice based on retroviral-mediated gene transfer of a dominant negative TGF-β type II receptor targeting murine bone marrow. Unlike the lymphoproliferative syndrome observed in TGF-β1-deficient mice, the disruption of TGF-β signaling in bone marrow-derived cells leads to dramatic expansion of myeloid cells, primarily monocytes/macrophages, and is associated with cachexia and mortality in lethally irradiated mice reconstituted with dominant negative receptor-transduced bone marrow. Surprisingly, there was a notable absence of T cell expansion in affected animals despite the observed differentiation of most cells in the T cell compartment to a memory phenotype. These results indicate not only that TGF-β acts as a negative regulator of immune function, but that lack of functional TGF-β signaling in the myeloid compartment of adult mice may trigger suppression of lymphocytes, which would otherwise proliferate when rendered insensitive to TGF-β. ***The Journal of Immunology,*** **2002, 169: 3485–3491.**

Transforming growth factor-β is a highly pleiotropic 25-kDa cytokine secreted by most cell types of the immune system and is known to play a variety of immunoregulatory roles, including the maintenance of lymphocyte homeostasis in vivo (1–3). Knockout mice deficient in TGF-β1 production show both embryonic and neonatal lethality as the result of a multifocal inflammatory response (4, 5), while TGF-β2- and TGF-β3-deficient mice suffer from a broad range of developmental defects (6–8). Transgenic mice with targeted disruptions of TGF-β signaling in T cells (9, 10) or B cells (11) display lymphocyte-mediated autoimmune pathology, and while these latter transgenic approaches have helped to elucidate the role of TGF-β signaling in individual leukocyte lineages, they leave open the question of immune pathology arising in adult mice as the result of TGF-β signaling perturbation in multiple leukocyte subtypes in adult animals. To study the effect of TGF-β on the cells of the immune system as a whole without compromising TGF-β signaling in peripheral tissues, it is necessary to isolate the effect of an experimental model to the hemopoietic compartment. Such an approach allows for the study of TGF-β immune regulation in the context of a host animal bearing normal TGF-β cytokine and receptor expression patterns elsewhere, insuring that TGF-β regulation of nonimmune processes, e.g., cell growth and differentiation, will be maintained.

TGF-β signaling is mediated through a pair of heterodimeric surface receptors, TGF-β type I and type II (12). The type II receptor provides a suitable target for disruption of the signaling pathway via a dominant negative receptor approach (13–15), as it is responsible for binding to activated soluble extracellular ligand, wherein it recruits the type I receptor into the signaling complex and initiates downstream signaling mediated by the Smad family of proteins (16–18). While TGF-β type II receptor knockout mice are nonviable due to defective yolk sac vasculogenesis in the embryo (19), targeted disruption of the TGF-β signaling pathway has been effectively achieved in a number of murine models by restricting the expression of a dominant negative type II TGF-β receptor (TBRIIDN)[3] in the tissue of interest, including the lymphocyte transgenic models discussed above as well as in nonlymphoid tissue such as the mammary gland (20) and pancreas (21). Therefore, we opted to disrupt TGF-β signaling by overexpressing a type II receptor construct with a truncated cytoplasmic domain in cells of the hemopoietic compartment through the use of retrovirally mediated gene transfer into murine bone marrow. Successfully infected murine bone marrow was then used to repopulate lethally irradiated adult C57BL/6 recipients, allowing for reconstitution of the host with TGF-β-insensitive leukocytes of all hemopoietic-derived subtypes (e.g., T cells, B cells, monocyte/macrophages, granulocytes, NK cells, and bone marrow-derived dendritic cells). Given the neonatal lethal phenotype of the TGF-β knockout mouse, we expected that a systemic inflammatory phenotype would develop in the reconstituted adult mice, deriving

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[3] Abbreviations used in this paper: TBRIIDN, dominant negative TGF-β type II receptor; CD62L, CD62 ligand; GFP, green fluorescent protein; HSC, hemopoietic stem cells; IRES, internal ribosomal entry sequence; MSCV, murine stem cell virus.

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primarily from lymphocyte-mediated autoimmunity. Unexpectedly, our results demonstrate that adult mice reconstituted with TGF-β-insensitive bone marrow develop dramatic expansion of myeloid, rather than lymphoid, cells in addition to a spontaneous differentiation of T cells from a naive to a memory phenotype, with mice developing marked cachexia within 3–4 mo posttransplant.

Materials and Methods

Construction of murine stem cell virus (MSCV)-TBRIIDN retroviral vector

The TBRIIDN was excised from PCDNA3-TBRIIDN by *Bam*HI/*Eco*RI digestion and inserted into the pMig-internal ribosomal entry sequence (IRES)-green fluorescence protein (herein designated MSCV-GFP) vector by first linearizing pMig with *Eco*RI and ligating in an *Eco*RI/*Bam*HI adapter (5′-AATTGGATCCGCGGCCGCG-3′, 3′-CCTAGGCGCCGGC GCTTAA-5′). These clones were designated MSCV-TBRIIDN and were screened by sequencing for correct orientation and insert number.

Production of retroviral supernatant

GP293 pantropic packaging cells (Clontech, Palo Alto, CA) were seeded at a density of 2.5×10^6 cells in T-25 collagen I-coated flasks (BIOCOAT; BD Biosciences, Mountain View, CA) 24 h before transfection in antibiotic-free DMEM/10% FBS. Cells were transfected using Lipofectamine Plus (Life Technologies, Gaithersburg, MD) with 2 μg pMig-TBRIIDN or pMig-GFP plus 2 μg vesicular stomatitis virus G plasmid for 12 h in serum-free medium and an additional 12 h after the addition of an equal volume of 20% FBS/DMEM. After 24 total h of transfection, the cells were washed gently in PBS, and fresh complete DMEM was added to the flasks, which were incubated for an additional 24 h before collection of supernatant.

Bone marrow isolation

Six- to 10-wk-old C57BL/6 (Ly5.2; Harlan Sprague-Dawley, Indianapolis, IN) or B6.SJL (Ly5.1; The Jackson Laboratory, Bar Harbor, ME) donor mice were anesthetized and injected i.p. with 5 mg 5-fluorouracil in 0.5 cc PBS. Five days later mice were sacrificed by cervical dislocation, and hind femora and tibiae were isolated and cleaned of muscle and soft tissue. Isolated bones were cut at the ends, and marrow was aseptically flushed in complete DMEM using 26-gauge needles (BD Biosciences) into 50-ml tubes, passed through a 40-μm pore size cell strainer (Falcon; BD Biosciences), and centrifuged at 500 × *g*. Pelleted cells were resuspended in 1× Pharmlyse (BD PharMingen, San Diego, CA) hypotonic ammonium chloride lysing solution to remove RBC from suspension and pelleted as described above before resuspension of cells at $1\text{–}2 \times 10^6$/ml in 24-well plates. Recombinant cytokines (R&D Systems, Minneapolis, MN) were added at concentrations of 20 ng/ml IL-3, 50 ng/ml IL-6, and 100 ng/ml stem cell factor and were replaced every 2 days of culture.

Infection of bone marrow culture and reconstitution of mice

After 48 h of culture, bone marrow cells were spun at 1000 × *g*, and supernatant was aspirated and replaced with infection mixture consisting of 1 ml viral supernatant, 10 μg/ml Polybrene (Sigma-Aldrich, St. Louis, MO), and HEPES buffer. Plates were centrifuged at 1000 × *g* for 90 min at room temperature, followed by addition of fresh cytokine-containing medium. This process was repeated at 72 h postisolation, followed by an additional 2 days of activation before transplant. Recipient C57BL/6 mice were irradiated in split doses of 800 and 400 rad, 3 h apart, in a Gammacell-40 irradiator (Atomic Energy of Canada, Mississauga, Ontario, Canada), and $1\text{–}2 \times 10^6$ cells were injected in PBS via warmed tail veins using 27-gauge needles. Transplant recipients were housed in pathogen-free facilities at the Center for Comparative Medicine, Northwestern University Medical School, and were maintained on trimethoprim/sulfamethoxazole for 4 wk after bone marrow transplant. All animal procedures were conducted under guidelines set by the animal care and use committee at Northwestern University Medical School.

Western blotting for Smad-2 phosphorylation

NIH-3T3 cells infected with pMig-TBRIIDN were trypsinized and collected in cold lysis buffer containing 1 mM Na_2VO_3 and centrifuged to remove cellular debris. Protein lysate was run on a Novex/10% acrylamide gel and blotted onto a polyvinylidene difluoride membrane. Blots were probed using anti-Smad2 (Upstate Biotechnology, Lake Placid, NY), anti-phospho-Smad2 (Upstate Biotechnology), or anti-GAPDH (Chemicon, Temecula, CA) mAb and visualized using ECL (Amersham Pharmacia Biotech, Piscataway, NJ) chemiluminescence kit.

Flow cytometric analysis of GFP expression in transplant recipients

Single-cell suspensions of bone marrow, spleen, or lymph nodes were obtained, and RBC were lysed as described above. The cells were resuspended in cold flow buffer (3% FBS and 0.1% sodium azide in PBS). All Abs and strepavidin-coupled fluorochromes were obtained from BD PharMingen, except as noted, and stained cell populations were analyzed for fluorescence on a FACSCalibur (BD Biosciences) in the Northwestern University Medical School Department of Microbiology/Immunology.

Results

Generation of retroviral vector and functional analysis of TBRIIDN

To develop a model of TGF-β signal down-regulation that affects all subclasses of leukocytes, but is strictly confined to cells of the

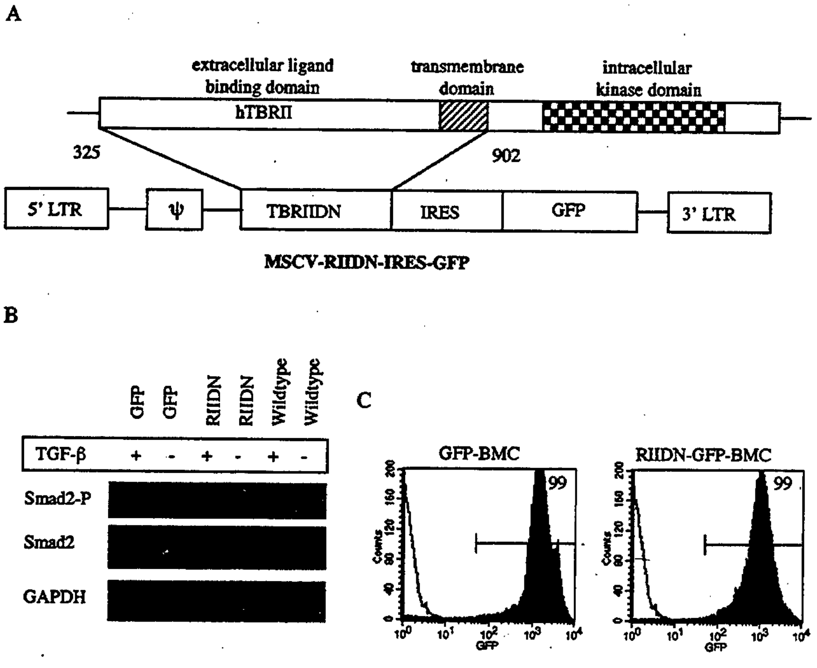

FIGURE 1. Structure, function, and expression of dominant negative receptor. *A*, Schematic diagram of retroviral construct. A truncated sequence of the human TGF-β type II receptor not containing the intracellular kinase signaling domain was cloned into the pMig vector to generate the MSCV-TBRIIDN vector. *B*, Functional analysis of infected primary mouse bone marrow cells indicates that addition of 10 ng/ml TGF-β abrogates phosphorylation of Smad-2 in TBRIIDN-transduced cells, but not in cells transduced with GFP control vector. Blots were stripped and reprobed with anti-Smad-2 and anti-GAPDH Abs as controls. *C*, FACS analysis of murine bone marrow cells 6 mo posttransplant. The results indicate long term robust expression of viral transgene in bone marrow of reconstituted mice receiving transplant of MSCV-infected donor cells.

hemopoietic compartment, we employed a retrovirally mediated gene transfer protocol targeting 5-fluorouracil-treated cultured murine bone marrow. As shown in Fig. 1*A*, we ligated a truncated sequence of the human TGF-β type II receptor into an MSCV-based bicistronic retroviral vector coexpressing GFP under the control of the 5′ long terminal repeat viral promoter (22, 23). The truncated receptor sequence contained both the extracellular ligand binding domain as well as the transmembrane domain, but lacks the cytoplasmic kinase domain responsible for mediating intracellular TGF-β signaling. Vesicular stomatitis virus G pseudotyped virus was generated in GP293 packaging cells, and the supernatant was used to infect ex vivo target cells cultured in IL-3, IL-6, and stem cell factor. Transfer efficiency into primary bone marrow cells using this approach was consistently 90% as assayed by GFP expression (data not shown), thus making it possible for us to forgo further FACS to obtain a high expressing population of donor cells. Functional analysis of the dominant negative receptor expressed in mouse bone marrow cells indicated that Smad-2 phosphorylation was absent in TBRIIDN-transduced cells treated with 10 ng/ml TGF-β in culture, but not in mock-infected cells or cells infected with vector controls expressing GFP alone (Fig. 1*B*), indicating specific abrogation of the TGF-β/Smad signal pathway in transgene-positive cells.

Characterization of viral transgene expression in hemopoietic lineages

To express the dominant negative receptor on all lineages of the hemopoietic compartment without affecting nonhemopoietic tissue, we isolated bone marrow from C57BL/6 (*Ptprc*^{b}, Ly5.2[CD45.2]) mice and transduced these cells ex vivo with the MSCV-TBRIIDN virus before reinfusion into lethally irradiated (1200 rad) C57BL/6 or congenic B6.SJL (*Ptprc*^{a}, Ly5.1[CD45.1]) recipients. Survival of GFP control bone marrow transfer recipients was >90% (21 of 22) at 6 mo post-transfer, confirming that the ex vivo culture protocol did not deplete the marrow of hemopoietic stem cells (HSC) (24, 25) or compromise the ability of the HSC to mediate long term radioprotection, and complete blood counts indicated comparable hematologic recovery of RBC, platelet, and WBC populations in both TBRIIDN mice and GFP controls (data not shown). Long term transgene expression in the bone marrow of transplant recipients was confirmed by flow cytometric analysis at 6 mo posttransplant, which indicated no significant reduction of GFP expression in either TBRIIDN or GFP-transduced mice (Fig. 1*C*). Expression of the viral progenome in hemopoietic lineages was assessed by flow cytometric analysis for GFP expression 2–3 mo after bone marrow transplant. As shown in Fig. 2, this regimen was effective in reconstituting both myeloid (Mac-1, Gr-1, CD11c) and lymphoid (CD3, B220, NK1.1) lineages with a high proportion of donor (GFP$^+$) cells. Transcriptional silencing in differentiating cell types, often a major concern in retroviral models of gene expression, was assayed by comparing CD45.2$^+$ (donor) and CD45.1$^+$ (recipient) expression on GFP$^-$ bone marrow cells and splenocytes. While both bone marrow and isolated splenocytes were repopulated almost entirely by GFP$^+$ cells (Figs. 1*C* and 2), the GFP$^-$ fraction of the spleen in both TBRIIDN and control mice was found to contain predominantly donor cells (data not shown), indicating a moderate loss of gene expression in maturing leukocytes, largely confined to the NK cell and T cell compartment.

TBRIIDN mice develop inflammatory response characterized by myeloid expansion

TBRIIDN-bone marrow transfer recipients showed no gross abnormalities for ~1–2 mo after bone marrow transplant, at which time both T and B cell development in the thymus and bone marrow, respectively, appeared phenotypically normal (data not shown); however, mice began to exhibit a progressive cachexic phenotype at time points between 2–3 mo (Fig. 3*A*), including ruffled fur, hunched posture, and dramatic weight loss of nearly 50% (26.4 ± 0.6 vs 14.4 ± 1.2 g) compared with littermate GFP controls ($p < 0.05$; $n = 10$/group). The mortality of mice receiving TBRIIDN-transduced bone marrow transplants was significantly increased compared with that of mice transplanted with marrow transduced with GFP vector controls (Fig. 3*B*). Because transgenic mice expressing a dominant negative TGF-β receptor specifically in T cells (10) displayed an autoimmune phenotype at

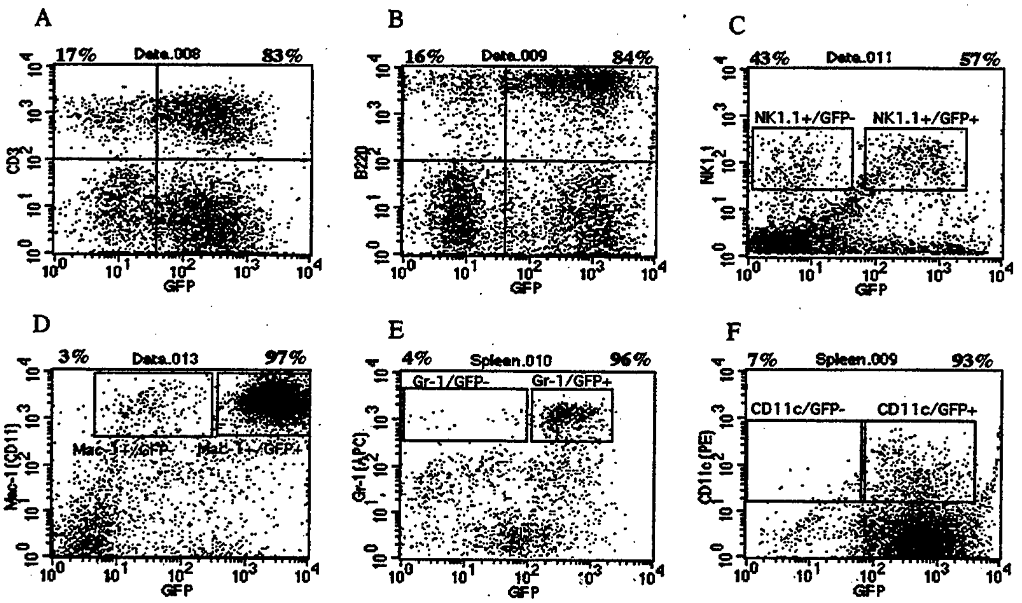

FIGURE 2. Multilineage expression of retrovirus progenome in spleens of bone marrow transplant recipients. Splenocytes from transplanted C57BL/6 mice were stained with anti-CD3 (*A*; T cells), anti-B220 (*B*; B cells), anti-NK1.1 (*C*; NK cells), anti-Mac-1 (*D*; macrophages), anti-Gr-1 (*E*; granulocytes), and anti-CD11c (*F*; dendritic cells) and were scored for the percentage of GFP-positive cells vs respective lineage marker.

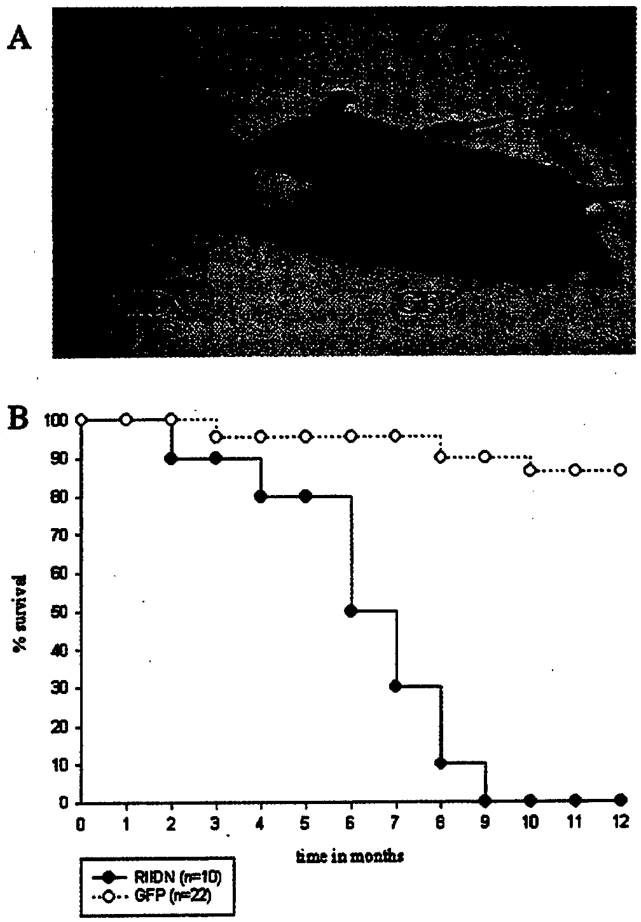

FIGURE 3. *A*, Morbidity in TBRIIDN mice. C57BL/6 mice reconstituted after lethal irradiation (1200 rad) with either TBRIIDN-transduced bone marrow (*left*) or GFP control vector-transduced marrow (*right*) ~5 mo after bone marrow transplant. *B*, Survival curve of TBRIIDN mice. Lethally irradiated mice transplanted with either GFP or TBRIIDN-transduced bone marrow were followed for survival for up to 12 mo. The results shown represent pooled data from three independent experiments ($p < 0.01$).

~6 mo of age, we suspected that the TBRIIDN bone marrow recipients could develop autoreactive immunity at an accelerated pace, given that all leukocyte subclasses were expressing the dominant negative receptor. To determine whether the observed cachexic phenotype was associated with lymphoproliferative disease, we analyzed splenocytes from affected mice for the expression of various lineage determinants and compared the total cell numbers and proportions of leukocyte subtypes to those for GFP controls. Flow cytometric analysis of splenocytes from TBRIIDN mice displaying cachexia revealed a dramatic expansion of a subpopulation of splenocytes displaying an altered forward/side scatter profile (Fig. 4). Analysis of these cells revealed that they were negative for lymphocyte cell surface markers (CD3/B220/NK1.1), suggesting that the expanded population was of myeloid origin. Indeed, staining of these cells for CD11b (Mac-1) indicated that monocytes/macrophages are probably the primary constituent of the expanded subpopulation (Fig. 4, *inset*), and total splenic Mac-1^{high} counts in TBRIIDN mice were 34.7×10^6 vs 7.8×10^6 for GFP controls ($p < 0.05$; $n = 3$/group). Histological analysis of TBRIIDN mice (Fig. 5) indicated a significant mononuclear infiltration into the extravascular tissue of the lungs, with an acute inflammatory infiltrate present in the bronchioles consisting primarily of polymorphonuclear cells, possibly due to leakage into the airspaces as the result of tissue damage around the alveolar spaces. The possibility of acute infection in the bronchiole appears unlikely given that all transplant recipients were maintained in pathogen-free barrier facilities for the duration of the experiment.

T cells differentiate to a CD25$^-$CD44$^+$CD62 ligand (CD62L)$^-$0 memory phenotype, but do not undergo proliferation in TBRIIDN mice

Transgenic mouse models of TGF-β insensitivity in T cells have indicated a spontaneous differentiation to a memory-like CD44^{high} phenotype in vivo. To investigate whether T cells derived from engrafted HSC expressing the viral TBRIIDN transgene spontaneously differentiated to an activated or memory phenotype, we examined the expression of activation markers CD44, CD62L, and CD25 (IL-2R). While levels of CD25 expression remained essentially unchanged between both groups throughout the experimental time course (data not shown), CD8$^+$ T cells recovered from spleens displayed a CD44^{high} phenotype (Fig. 6) as early as 6 wk post-transplant, consistent with the transgenic models discussed above. While CD44 up-regulation was an early event, usually taking place before the onset of obvious morbidity, CD62L down-regulation appeared to be a temporally independent event and was typically not observed in either CD4 or CD8 T cells before 3–4 mo of age (data not shown), usually well after the onset of the cachexic phenotype. In older TBRIIDN mice, CD62L was down-regulated significantly (Fig. 6) on both CD4/CD8 cells, but total T cell counts recovered from the spleens of highly moribund mice were not elevated over those of control mice (data not shown).

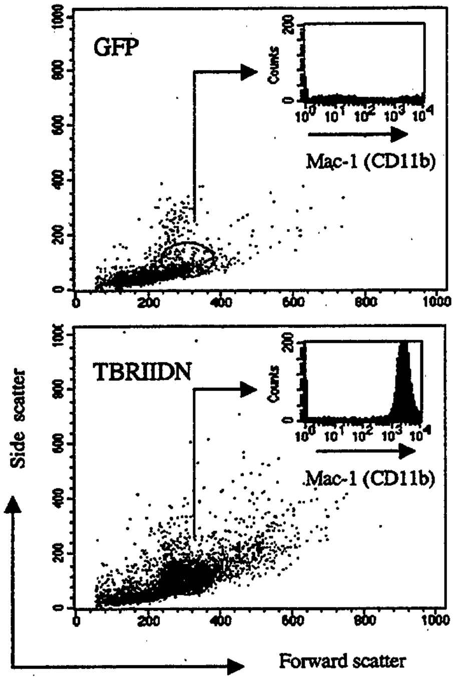

FIGURE 4. Expansion of Mac-1$^+$ cells in splenocytes of reconstituted mice. Splenocytes were isolated from mice 60 days after bone marrow transplant and were stained for various cell surface markers. Results from mice transplanted with MSCV-IRES-GFP (*A*) or MSCV-TBRIIDN-IRES-GFP-transduced bone marrow (*B*) after lethal (1200 rad) irradiation are shown. The forward/side scatter profile indicates the expansion of a subpopulation of splenocytes in the TBRIIDN mouse, but not in the GFP mouse; gating on this population as shown indicates that the expanded population is positive for the myeloid marker Mac-1 (*inset*).

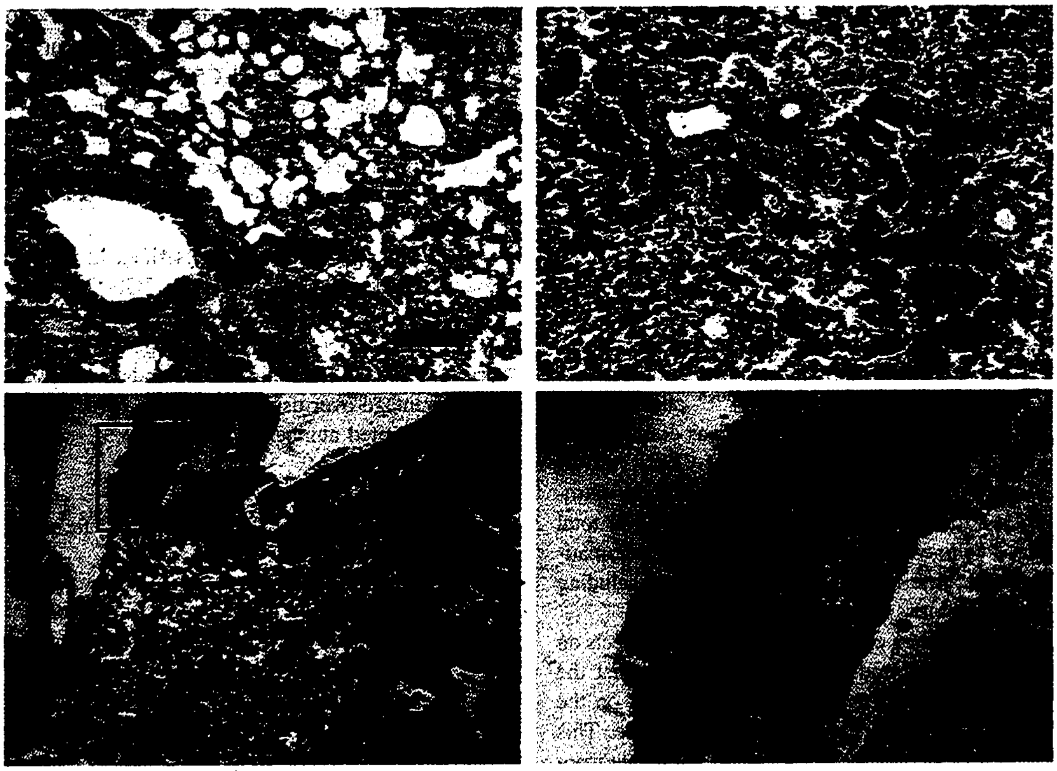

FIGURE 5. Histologic manifestations of inflammation in TBRIIDN transplanted mice. H&E staining of paraffin-embedded lung sections from a GFP control mouse (*A*) and a TBRIIDN mouse (*B*), indicating perivascular mononuclear and polymorphonuclear inflammatory infiltrate as well as disruption of normal alveolar architecture. Scale bars: *A* and *B*, 100 μm; *C*, 200 μm; *D*, 50 μm.

Discussion

Using retrovirally mediated-gene transfer targeting HSC, we investigated the consequences TGF-β insensitivity limited to hemopoietic tissue, but not limited to expression on a single leukocyte subtype, and characterized the gross pathology of the resulting immune syndrome in mice. Lethally irradiated C57BL/6 mice reconstituted with bone marrow expressing TBRIIDN exhibited immune-mediated pathology manifested by inflammation of peripheral tissue and a gradual cachexic phenotype, leading to significantly increased mortality.

The retroviral gene transfer approach allowed us to examine the role of TGF-β signaling in immune homeostasis only so far as it involves the immune compartment and eliminated the possible contribution of other tissues to the observed phenotype, as is the concern with non-tissue-specific cytokine knockouts. For example, TGF-β1 knockout mice show aberrant expression of MHC II on nonimmune tissue (26), possibly contributing to the autoimmune inflammatory phenotype. The cytokine knockout approach also leaves open the possibility of TGF-β signaling through redundant activity mediated by TGF-β2 or TGF-β3, which may account for the phenotypic difference seen between the autoimmune phenotype of the TGF-β1 knockout mouse and the nonviability of the TGF-β type II receptor knockout.

The major technical concern in generating a model of TGF-β insensitivity using retroviral targeting of HSC is that mature leukocytes derived from primitive transduced precursors will exhibit transcriptional silencing of the transgene due either to multiple stages of differentiation involving chromosome remodeling or perhaps as a function of time (27). In the model described here there was little if any silencing of the viral progenome as assayed by flow cytometry for GFP expression in the unfractionated bone marrow of reconstituted host mice, with the GFP$^+$ fraction typically 95%. Although there was evidence of limited transgene silencing in the mature leukocytes of TBRIIDN-reconstituted mice,

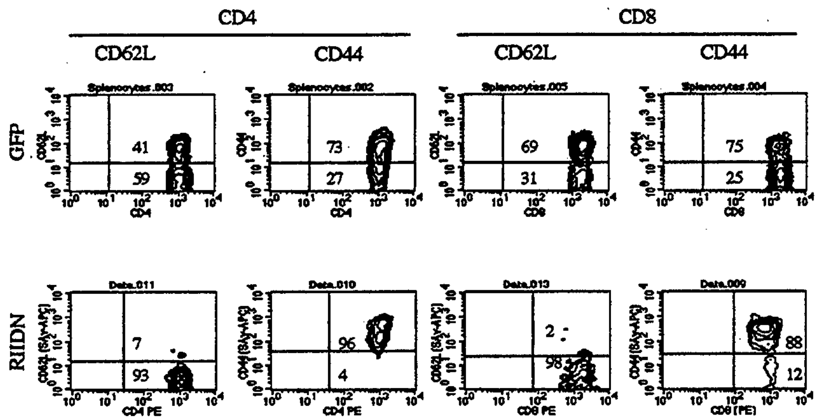

FIGURE 6. T cell phenotype analysis of TBRIIDN reconstituted mice vs GFP controls. CD4 or CD8 T cells were analyzed for CD44 and CD62L (L-selectin) expression via flow cytometry. Data are representative of mice analyzed at 6 mo after bone marrow transplant.

it appears that the change in the activation profile of T cells to a memory (CD44^{high}CD62^{low}) phenotype was not absolutely dependent on maintaining transgene expression for the life span of the individual cell; rather, it appears that early phenotypic changes occurred before down-regulation of the TBRIIDN or, alternatively, that differentiation to a memory phenotype resulted from activation pathways mediated by other leukocytes rendered insensitive to TGF-β. It is notable that there was an absence of dramatic CTL proliferation even among those mice exhibiting the most acute symptoms, suggesting that lymphoproliferation in the above mentioned T cell transgenic mice may involve a complex regulatory pathway that is, in fact, inhibited in the context of overall immune TGF-β insensitivity, a surprising finding given the TGF-β knockout phenotype. The differentiation to a CD44^{high} phenotype in TBRIIDN T cells of both CD4 and CD8 lineage was dramatic and essentially total. Decreased surface expression of CD62L (L-selectin), typical of memory T cells, was not observed in our retroviral model until 3–4 mo after bone marrow transfer. The total number of splenic T cells was not observed to increase dramatically at any point in the time course of the experiment, including after the development of gross abnormalities in the mice.

TGF-β is noted to exert often contradictory regulatory effects on numerous leukocyte lineages, and it has been observed that the effect of TGF-β on a given cell type is often dependent on the overall cytokine milieu in which the signaling takes place. This also appears to be the case for TGF-β signaling in monocyte/macrophages, where the balance between inflammatory cytokines such as IFN-γ and TGF-β may direct M-CSF-dependent bone marrow precursors toward either an osteoclastic or a cytocidal response to TNF-α, respectively (28). This model suggests that in the absence of TGF-β signaling, as is the case with TBRIIDN-bearing precursor cells, M-CSF-dependent precursors may be biased toward an IFN-γ-responsive cytocidal pathway, which could help explain the dramatic expansion of Mac-1^{high} mononuclear cells in lymphoid tissue and peripheral blood reported here. Further studies in our laboratory are currently being conducted on mouse models deficient in various proinflammatory cytokines to determine whether mediation of the observed wasting phenotype is critically dependent on macrophage-secreted products such as TNF-α. Another line of investigation underway seeks to determine whether inducible NO synthase production of NO by activated macrophages may mediate an anti-proliferative effect on the T cell compartment in our model, which is suggested by up-regulation of inducible NO synthase in TGF-β1 knockout mice (29, 30) as well as by established mechanisms of NO-mediated T cell suppression in infectious disease (31) and cancer (32). Furthermore, the flexibility of the retroviral approach will allow us to examine phenotypes generated by reconstitution with TBRIIDN-transduced bone marrow in transgenic mice deficient in T cell-mediated immunity, which may help to define the role of Ag-specific immune responses in the pathology described here.

The issue of TGF-β regulation of stem cell differentiation is open to further study by the use of this retroviral model, and it must be considered a possibility that the absence of functional TGF-β signaling in bone marrow precursors could introduce a developmental bias in the normal differentiation program from primitive, uncommitted precursor cells to myeloid or lymphoid lineage-committed progenitors. It is clear from our data that expression of TBRIIDN does not preclude HSC engraftment or multilineage reconstitution of the hemopoietic compartment, but this does not address the issue of development per se, other than to indicate that there is no obvious block of development in any one particular leukocyte lineage. TGF-β has been hypothesized to act as a critical regulator of HSC growth and cell cycle regulation (33–36) via its effects on cyclin-dependent kinase inhibitors (37–39) and may play a pivotal role in the maintenance of a quiescent stem cell pool in vivo. TBRIIDN-repopulated C57BL/6 mice lose their ability to repopulate lethally irradiated mice in a serial transplant assay (A. H. Shah, W. B. Tabayoyong, and C. Lee, unpublished observations), while defects in hemopoiesis and vasculogenesis have been reported in both cytokine (40) and type II receptor (19) transgenic mice.

We believe that the model described here offers a useful approach to define the in vivo role of TGF-β in immune regulation and hemopoiesis. The facts that the model is based on wild-type mice and can be easily modified to generate similar models on a variety of available genetic backgrounds give this approach a practical advantage over using transgenics, which are typically available in a limited number of strains.

Acknowledgments

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Advances in Brief

Suppression of Tumor Metastasis by Blockade of Transforming Growth Factor β Signaling in Bone Marrow Cells through a Retroviral-mediated Gene Therapy in Mice[1]

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Abstract

Transforming growth factor B (TGF-β) is a potent immunosuppressive cytokine that is frequently associated with mechanisms of tumor escape from immunosurveillance. We report that transplantation of murine bone marrow (BM) expressing a dominant-negative TGF-β type II receptor (TβRIIDN) leads to the generation of mature leukocytes capable of a potent antitumor response *in vivo*. Hematopoietic precursors in murine BM from donor mice were rendered insensitive to TGF-β via retroviral expression of the TβRIIDN construct and were transplanted in C57BL/6 mice before tumor challenge. After i.v. administration of 5×10^5 B16-F10 murine melanoma cells into TβRIIDN-BM transplanted recipients, survival of challenged mice at 45 days was 70% (7 of 10) *versus* 0% (0 of 10) for vector-control treated mice, and surviving TβRIIDN-BM mice showed a virtual absence of metastatic lesions in the lung. We also investigated the utility of the TGF-β-targeted approach in a mouse metastatic model of prostate cancer, TRAMP-C2. Treatment of male C57BL/6 mice with TβRIIDN-BM resulted in the survival of 80% (4 of 5) of recipients *versus* 0% (0 of 5) in green fluorescent protein-BM recipients or wild-type controls. Cytolytic T-cell assays indicate that a specific T-cell response against B16-F10 cells was generated in the TβRIIDN-BM-treated mice, suggesting that a gene therapy approach to inducing TGF-β insensitivity in transplanted BM cells may be a potent anticancer therapy.

Introduction

Tumor immunotherapies to date have focused largely on the priming of immune responses to fight cancer, with mixed results and generally poor efficacy. In addition to immune stimulation, the issue of overcoming active immune suppression must also be considered when developing an immune-based strategy for cancer therapy (1, 2), particularly with regard to secreted soluble factors that are known to down-regulate immune function and antitumor response. Most significant of these is the pleiotropic cytokine TGF-β,[3] (3) which has previously been shown to act in a critical inhibitory fashion on most cells of the immune system and is secreted by a wide variety of tumor types, many of which down-regulate expression of their own TGF-β receptors (4–7) to circumvent the growth-inhibitory activity of TGF-β signaling. Tumor-secreted TGF-β is capable of inhibiting the response of tumor-specific lymphocytes (8), including sites of metastatic tumor growth (9). The potency of TGF-β as an immunosuppressive cytokine makes it an attractive target as an anticancer therapy, because, as it has been suggested, a breakdown of self-tolerance mechanisms in the periphery may be a critical element in fighting nonimmunogenic tumors (10). We hypothesized that an immunotherapy strategy that specifically blocks TGF-β signaling in immune cells, regardless of tumor location or tumor microenvironment, could be highly successful in mediating an antitumor response.

We chose to use a retroviral-mediated gene therapy approach abrogating TGF-β signaling in hematopoietic stem cells in the BM, because this approach has been shown recently to be a successful protocol in the delivery of long-term transgene expression in immune effector cells (11). Here, we show that abrogation of TGF-β signaling in the immune compartment via retrovirus-mediated expression of a TβRIIDN in transplanted BM-derived stem cells elicits potent antitumor activity when treated animals are challenged i.v. with highly tumorigenic melanoma or prostate cancer cells.

Materials and Methods

Mice. Male C57BL/6 mice, 6–8 weeks of age, were obtained from Jackson Labs (Bar Harbor, ME) and maintained in pathogen-free facilities at the Center for Comparative Medicine at Northwestern University Feinberg School of Medicine in accordance with established guidelines of the Animal Care and Use Committee of Northwestern University.

BM Isolation and Culture. Donor mice were inhalation-anesthetized and were given injections i.p. of 5 mg of 5-fluorouracil (Sigma, St. Louis, MO). Five days later, mice were sacrificed by cervical dislocation and hind femora and tibiae were isolated and cleaned of tissue before being flushed aseptically with DMEM plus 10% fetal bovine serum (DMEM-10) using 26-gauge needles. The RBCs in the marrow preparation were then lysed using a hypotonic ammonium chloride solution (PharMingen, Becton-Dickinson, San Diego, CA). The processed marrow was resuspended in fresh DMEM-10 supplemented with 100 ng/ml stem cell factor, 50 ng/ml IL-6, and 20 ng/ml IL-3 (R&D, Minneapolis, MN) at $1\text{–}2 \times 10^6$ cells/ml, and were incubated at 37°C/5% CO_2.

Construction of TβRIIDN-GFP Retroviral Vector. The procedure for the construction of the TβRIIDN viral vector has been described earlier (12). Briefly, a truncated sequence of the human TGF-β type II receptor was cloned into a mouse stem-cell virus-based bicistronic retroviral vector coexpressing GFP under the control of the 5′ long terminal repeat viral promoter. The truncated receptor contained both the extracellular domain and the transmembrane domain but lacked the cytoplasmic kinase domain. The control empty vector was designated as the GFP vector.

Production of Infectious TβRIIDN-GFP Retrovirus. Pantropic GP293 retroviral packaging cells (Clontech, San Diego, CA) were seeded at a density of 2.5×10^6 cells in collagen-I-coated T-25 flasks (BIOCOAT; BD Biosciences, Mountain View, CA) 24 h before plasmid transfection in antibiotic-free DMEM-10, such that the cells were ~70–90% confluent at the time of

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[3] The abbreviations used are: TGF-β, transforming growth factor β; GFP, green fluorescent protein; TβRIIDN, dominant negative type II TGF-β receptor; BM, bone marrow; TRAMP, TGF-β-targeted approach in a mouse metastatic model of prostate cancer; IL, interleukin.

transfection, at which point the cells were rinsed with PBS to remove residual serum. A mixture of 2 μg of retroviral plasmid and 2 μg of VSV-G envelope plasmid were cotransfected in serum-free DMEM using LipofectAMINE-Plus (Invitrogen, Gaithersburg, MD) according to the manufacturer's protocols with the following modifications. Cells were transfected for 12 h followed by the addition of an equivalent volume of DMEM-20 and reincubation for an additional 12 h. After 24 h of total transfection time, the supernatant was aspirated, the cells were rinsed gently in PBS, and 3 ml of fresh DMEM-10 was added to each flask. After 24 h, virus-containing supernatant was collected and used to infect target cells.

Western Blotting for SMAD-2 Phosphorylation. The infected primary mouse BM cells were treated with or without 10 ng/ml TGF-β1 for 30 min in culture to test the functionality of the TGFβ signaling pathway (12). Proteins in the cell lysate were subjected to electrophoresis (Novex/10% acrylamide gel) and blotted onto a polyvinylidene difluoride membrane. Blots were probed using monoclonal antibody against phosphorylated *SMAD-2*. Blots were stripped and reprobed with antibodies against *SMAD-2* and then glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Retroviral Infection and Transplantation of Murine BM. Cultured murine BM cells were infected on days 2 and 3 postisolation via spin infection as follows: an aliquot of 1 ml of viral supernatant was added to each well of a 24-well plate containing BM cells in the presence of a minimum concentration of 4 μg/ml Polybrene (Sigma), spun at 1000 × *g* for 90 min, and supplemented with 1 ml of fresh cytokine-supplemented DMEM-10. On day 4–5, cells were examined for GFP expression, washed two times in PBS, and injected into the lateral warmed tail veins of irradiated (1200 rads) recipient C57BL/6 mice. Transplanted mice were maintained on sulfamethoxazole/trimethoprim for a minimum of 2 weeks to prevent opportunistic infection.

i.v. Inoculation of Tumor Cells into Mice after BM Transplant. C57Bl/6 mice receiving TβRIIDN, GFP, or nontransduced BM transplants were challenged i.v. with 5×10^5 B16-F10 cells ($n = 10$ mice/group) or TRAMP-C2 cells ($n = 5$ animals/group) 2 months after transplant. The B16-F10-challenged mice were monitored for morbidity and mortality for 6 weeks, and the TRAMP-C2-challenged mice were monitored for 8 weeks. At the conclusion of each experiment, all of the animals were inspected for the presence of metastases. Statistical analysis was conducted on a Kaplan-Meier survival curve, using the log-rank test (13).

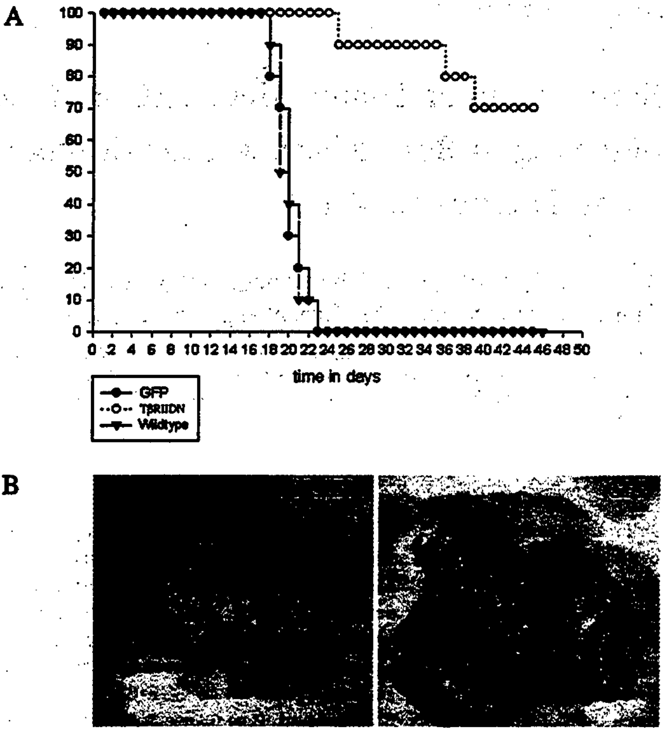

Fig. 1. Antitumor capacity of mice receiving transplant of TβRIIDN-BM. *A*, Kaplan-Meier survival curve of C57BL/6 mice challenged with 5×10^5 B16-F10 melanoma cells via tail vein injection after transplantation with $2–4 \times 10^6$ syngeneic BM cells transduced with TβRIIDN-expressing retrovirus, GFP control virus, or uninfected wild-type BM cells ($n = 10$/group; $P < 0.01$ by the log-rank test for the TβRIIDN group *versus* GFP or control group; Ref. 13). *B*, lungs of mice 3 weeks post-tumor challenge from TβRIIDN-transplanted mice (*left*) or GFP control mice (*right*). The GFP control lung is covered with black, melanin-producing tumor cells. The lung in the TβRIIDN-treated group is devoid of any tumor.

Results

Functional Status of TGF-β Signaling in Transfected BM. Transfection efficiency into primary BM cells using the above approach was consistently greater than 90% as assayed by GFP expression (12). Results of the functional analysis of these transfected BM cells have been reported earlier (12). Briefly, when the TβRIIDN BM cells were treated with 10 ng/ml TGF-β1 in culture, the expression of the dominant negative receptor resulted in an absence of *SMAD-2* phosphorylation. *SMAD-2* phosphorylation was observed in similarly treated mock-infected cells or cells infected with the control vector expressing GFP alone (12). Furthermore, at 6-months posttransplant, the results of flow cytometry data indicated that there was no significant reduction of GFP expression in BM cells of either TβRIIDN- or GFP-transduced mice (12).

Increased Survival and Decreased Metastases in TβRIIDN-BM-treated Mice. C57BL/6 mice receiving TβRIIDN, GFP, or nontransduced BM transplants ($n = 10$ mice/group) were challenged with 5×10^5 B16-F10 cells i.v. and monitored for morbidity and mortality for a period of ~6 weeks. Whereas 100% of wild-type and GFP transplant recipients were dead by 22 days postchallenge, there was no mortality observed in the TβRIIDN-BM recipient group (Fig. 1*A*) by this time. The TβRIIDN-BM control group was monitored for a total period of 45 days postchallenge, at which point surviving (7 of 10) mice were sacrificed and their lung tissue removed for macroscopic examination to determine whether metastatic lesions comparable with those observed in the wild type-BM and GFP-BM control groups were present. As shown in Fig. 1*B*, the lung tissue of untreated control mice was characterized at the time of death by numerous black melanoma metastases throughout the tissue. However, the TβRIIDN-BM-treated group had fewer metastatic lesions in the lungs of nonsurviving mice and virtually no discernable lesions in the lungs of mice surviving throughout the duration of the experiment. These results strongly suggest that mice transplanted with BM with targeted blockade of TGF-β signaling generate potent antitumor immunity in C57BL/6 mice challenged with highly metastatic, nonimmunogenic tumor cells.

To determine the efficacy of the TβRIIDN-BM treatment on metastatic tumor formation in a model of prostate cancer, we subsequently challenged TβRIIDN-BM treated male C57BL/6 mice with i.v. administration of 5×10^5 TRAMP-C2 cells and monitored the mice similarly as described above. At 3 weeks postchallenge, macroscopic tumor formation was difficult to detect in either the treated or untreated controls, indicating that the TRAMP-C2 tumor cells were not as aggressive in their formation of metastatic lung foci as were the B16-F10 tumor cells. However, on further examination of histological specimens of mice sacrificed at 21 days post tumor challenge, micrometastatic lesions were already visible in the GFP group but not in the TβRIIDN group (data not shown). A second group of mice was tumor challenged and monitored for a period of 8 weeks, by which point the survival of the wild-type and GFP control mice was 0% (0 of 5, each group by week 7; Fig. 2*A*), whereas the survival of the TβRIIDN-BM treated cohort was 100% (5 of 5). By week 9, one animal in the TβRIIDN-BM group died, leaving the overall survival rate of 80% (4 of 5) for this group. Results of statistical analysis, using the log-rank test, indicated $P < 0.05$ between the TβRIIDN-BM and the other two control groups. Postmortem analysis of the untreated or vector-control-treated animals indicated a significant tumor burden evident in the lung tissue of each mouse (Fig. 2*B*), whereas the lungs of

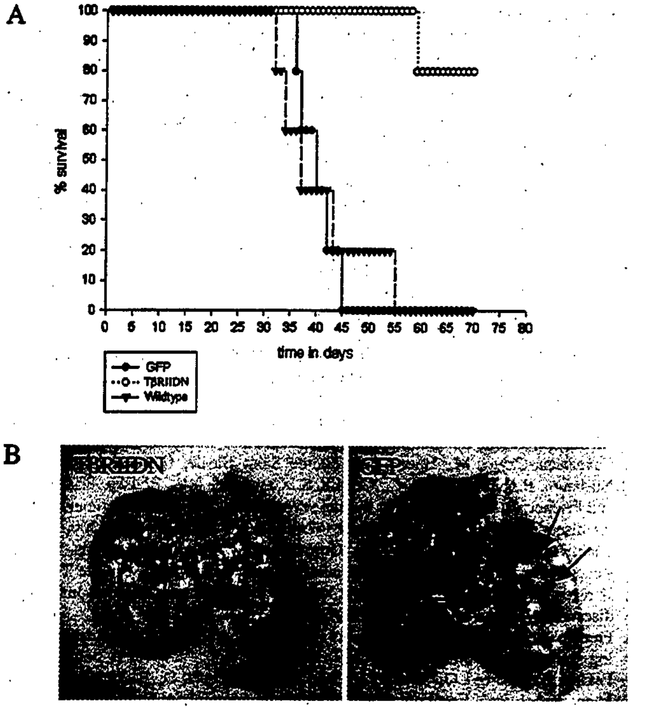

Fig. 2. TβRIIDN-BM-treated mice showing antitumor capacity against TRAMP-C2 mouse prostate cancer tumor challenge. 5×10^5 TRAMP-C2 prostate adenocarcinoma cells were injected via the tail vein into TβRIIDN-BM-treated mice, and the mice were monitored for morbidity and mortality. *A*, survival of wild-type (untreated), GFP, and TβRIIDN-transplanted mice post-tumor challenge (n = 5/group), expressed as the Kaplan-Meier curve. ($P < 0.05$ by the log-rank test for the TβRIIDN group *versus* the control or GFP group; Ref. 13). *B*, lung tissue from TβRIIDN-BM- and GFP-BM-treated mice at 6 weeks post-tumor challenge indicating metastatic tumor foci (*arrows*).

TβRIIDN mice remained metastases free. From these data, we conclude that targeting immune TGF-β signaling with BM-directed retroviral therapy is an effective means of preventing metastatic prostate tumor growth in mice.

TβRIIDN Mice Generate Specific Antitumor CTLs *in Vivo*. To determine whether the antitumor response generated by transplant of TβRIIDN-BM is tumor-specific, we collected splenocytes from TβRIIDN-BM- and GFP-BM-tumor-challenged mice at 3 weeks post-tumor challenge and assayed the ability of CTLs to lyse B16-F10 cells *in vitro* using a standard ^{51}Cr release assay. Results from the CTL assay indicated a significant increase in tumor-specific lysis of melanoma cells in splenocytes from TβRIIDN-BM-transplanted mice compared with GFP control-treated counterparts (Fig. 3*A*), suggesting that the antitumor phenotype in TGF-β signaling pathway-deficient mice is at least partially caused by CTL activity and not simply a result of broader, nonspecific immune stimulation of treated mice. Likewise, a ^{51}Cr release assay performed on labeled TRAMP-C2 cells by splenocytes recovered from TβRIIDN-BM- and GFP-BM-transplanted mice (Fig. 3*B*) indicate that tumor-specific cytolysis is generated by the retroviral blockade of TGF-β signaling.

Discussion

Results of the present study demonstrate that disruption of the TGF-β signaling pathway in BM cells using a gene therapy approach confers an antitumor phenotype on treated mice. Targeting of TGF-β-mediated immunosuppression has been used previously to show that the blockade of normal TGF-β signaling pathways confers an antitumor effect in a variety of tumor models, either via modulation of tumor TGF-β production in a tumor vaccine approach or via the systemic down-regulation of available TGF-β cytokine in the serum, and has been used in a variety of tumor therapies to combat both primary and secondary tumor growth. *Ex vivo* transfer of an antisense TGF-β construct into isolated tumor cells followed by reimplantation into the brain of rats with established gliomas has been shown to result in complete eradication of the tumors *in vivo* (14), and a similar approach has been used successfully to confer immunogenicity to a prostate tumor model in the Dunning rat (15). Systemic administration of anti-TGF-β antibody and IL-2 shows a significant decrease in number and size of metastatic B16 tumor lesions (16), suggesting that TGF-β immunosuppression can be at least partially overcome by a general TGF-β signal blockade. This latter approach, including similar approaches such as soluble TGF-β type II receptor therapy (17), although providing a rationale for a TGF-β-targeted approach in cancer therapy, may be ultimately limited in its ability to mediate antitumor effects at sites in which the delivery of a soluble therapeutic agent may be insufficient to block TGF-β present at high concentrations in tumor microenvironments.

In the present study, we demonstrated the therapeutic efficacy of targeting progenitors of leukocyte populations in the BM with retroviral particles that specifically blocked TGF-β signaling by expressing a dominant negative TGF-β type II receptor with a truncated cytoplasmic domain. The lack of formation of metastatic lesions in TβRIIDN-BM-treated mice after i.v. administration with highly metastatic B16-F10 cells emphasizes the importance of the TGF-β signaling pathway to tumorigenicity *in vivo*, even in the case of tumor cells with aggressive growth properties and little natural immunogenicity. Likewise, the lack of metastatic lesion formation in TβRIIDN-

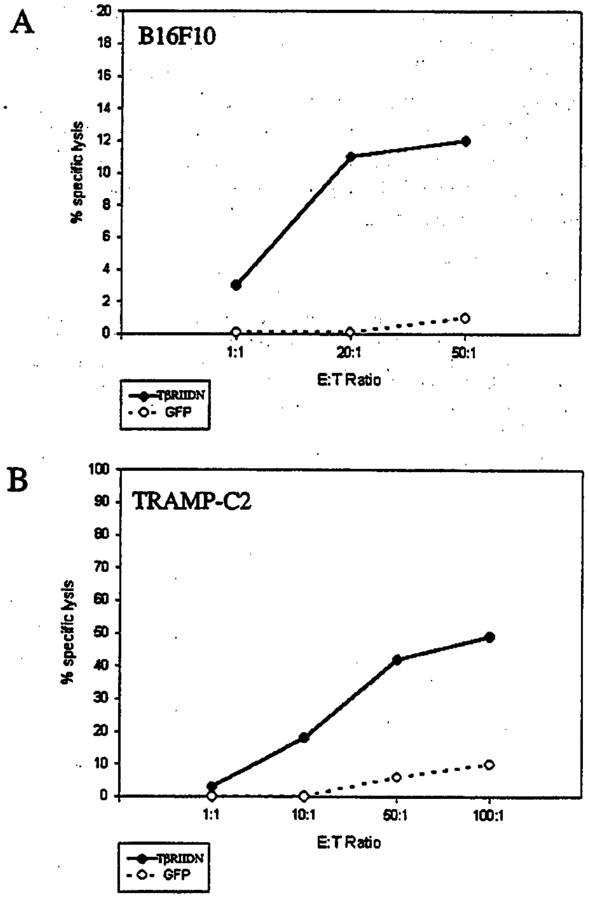

Fig. 3. Generation of tumor-specific killing in TβRIIDN-BM-transplanted mice. Splenocytes from tumor-challenged mice were collected and stimulated for 5 days with irradiated B16-F10 mouse melanoma cells (*A*) or with TRAMP-C2 mouse prostate carcinoma cells (*B*) before being cocultured with ^{51}Cr-labeled targets at the indicated E:T ratios. Samples were analyzed in duplicate (*A*) or triplicate (*B*) wells.

BM-treated animals after a challenge with TRAMP-C2 cells, a murine model of prostate cancer, supports the idea that this antitumor approach is viable in a range of cancers of different tissue origins.

The potency of TGF-β as an immunoregulatory cytokine that is critical for the maintenance of immune homeostasis also necessitates the careful application of perturbations in the TGF-β signaling processes for cancer immunotherapy. The potential for the generation of widespread autoimmunity and inflammation, which is generated in the absence of functional TGF-β pathways in immune cells (12), makes it essential that the approach described here be maximized for its utility as an antitumor therapy but modified so as to minimize potential autoimmune side effects against host tissue. Mice that are deficient in TGF-β1 cytokine display a massive auto-inflammatory phenotype and quickly succumb to systemic damage in a variety of tissues (18, 19), whereas other transgenic models, restricted to TGF-β-signal abrogation in the immune compartment or single lineages including T (20) and B cells (21), similarly result in dysregulation of immune function. The retroviral approach to therapeutic gene delivery can be enhanced by vectors that offer a regulatory mechanism to control expression of the transgene and/or survival of transgene-positive cells, whether through the use of on/off systems responsive to pharmacological agents (*e.g.*, tetracycline) or through the use of suicide gene elements present in the integrated viral genome.

We submit that the results presented here represent a viable approach to the problem of tumor escape from immune surveillance using readily available retroviral gene transfer technology, and we suggest that this approach could potentially be coupled with other immunostimulatory protocols that generate tumor-specific lymphocyte responses but that, to date, have had only mixed results because of a lack of cytotoxic effector activity, particularly with regard to distant metastatic tumor foci, as a result of TGF-β-mediated immunosuppression. The hematopoietic stem-cell gene therapy approach, already established as a viable means for the delivery of therapeutic genes to cells of the immune system, provides a legitimate and characterized target for TGF-β signaling-directed therapy for a potentially wide variety of cancers.

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From TGF-β to Cancer Therapy

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Abstract: This article will introduce a novel concept in the use of TGF-β insensitive host immune cells in cancer therapy. TGF-β is a multi-functional cytokine. At a cellular level, it mediates cellular proliferation, growth arrest, differentiation and apoptosis. Because of the above cellular effects, TGF-β is able to regulate a host of patho-physiological events *in vivo*, such as normal embryonic development, angiogenesis in tumor tissues, malignant transformation and immune surveillance.

As a general rule, its direct effect on cancer cells is inhibition to cancer growth. However cancer cells are able to acquire the ability to evade this inhibitory effect of TGF-β by becoming insensitive to TGF-β. Furthermore, these malignant cells are able to produce large quantities of TGF-β. The consequence of over expression of TGF-β by cancer cells is an important factor for subsequent tumor progression. The excess amount of TGF-β promotes tumor angiogenesis and immune suppression. The latter effect of TGF-β is the most devastating to the host. The present discussion is focused on the role of TGF-β insensitive immune cells in cancer growth.

The host immune system offers a natural defense program against cancer. But, this natural immune surveillance is rendered ineffective by an overproduction of TGF-β derived from the tumor cells. Rendering the host immune cells insensitive to TGF-β in a gene therapy program offers a hope for us to successfully combat against cancer. Based on the above discussion, it is encouraging that there is a possibility for us to achieve a cure in cancer using TGF-β insensitive immune cells in gene therapy.

Key Words: Transforming Growth Factor-beta (TGF-β), cancer therapy, TGF-β receptors (TβR-I, TβR-II, TβR-III), TGF-β insensitive immune cells, Serine/threonine kinases, Cycline-dependent kinases (cdks), Myc Family members, Smad's family.

1. INTRODUCTION

Cancer cells have eluded the minds of the most brilliant scientists. American Cancer Society estimates that, in 2002, more than a million and quarter men and women in the US will be diagnosed with cancer and more than half a million will die of this disease [1]. Recent advances in the area of cancer gene therapy and immunotherapy have been encouraging. The development of the targeted therapeutic strategy has saved many lives. Despite these promises, the overall survival rate for cancer patients has been disappointing [2, 3]. In this review, we present an intriguing biology of transforming growth factor-beta (TGF-β), which offers a hope for our fight against cancer. The present review will introduce a novel concept in the use of TGF-β insensitive host immune cells in cancer therapy.

2. BASIC BIOLOGY OF TGF-β

Historically, TGF-β was recognized as a growth factor for murine sarcoma virus-transformed rat kidney fibroblasts, as it promoted soft agar anchorage-independent grow [4]. This original understanding of TGF-β has been modified. Today, we recognize that TGF-β is a multifunctional growth factor, which regulates a wide array of events in patho-physiology.

The TGF-β Superfamily

TGF-β is the prototypic member of a superfamily that consists of more than 30 members. Some of the members include inhibin, bone morphogenic proteins (BMPs), and Mullerian inhibiting substance. The TGF-β subfamily contains five members (TGF-β1, -β2, -β3, -β4, and -β5). TGF-β4 and -β5 have been identified only in chicken and Xenopus, respectively [5-7]. TGF-β1, -β2, and -β3 have been identified in mammals [8, 9]. Although TGF-β is a pleiotropic growth factor, it is mainly a growth inhibitor to most cell types [10, 11]. This family of growth factors regulates pivotal biological functions, including cell proliferation, differentiation, apoptosis, migration, and extracellular matrix production [12, 13].

Biochemistry of Mammalian TGF-β

TGF-β is synthesized as the C-terminal domain of a precursor form that is cleaved before secretion from the cell [8, 14, 15]. However, the TGF-β pro-peptide, which is

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referred to as the latency associated peptide (LAP), non-covalently bound to TGF-β after secretion and cannot bind to β-glycan or the signaling receptors [16]. Most cell types secrete TGF-β in this biologically inert form. A third component of the latent TGF-β complex is a large secretory glycoprotein known as latent TGF-β -binding protein (LTBP), which is disulfide-linked to LAP. LTBP is implicated in TGF-β secretion, storage in the extracellular matrix, and eventual activation [17].

TGF-β Receptors

TGF-β exerts its biological effect through its receptors. In animal cells, three types of TGF-β receptors have been reported as type I, II and III receptors (TβR-I, TβR-II, TβR-III) [18]. TβR-III is a 200-400-kD proteoglycan, with chondroitin sulfate and heparan sulfate chains linked to a 110-130 KD core protein [19]. TβR-III has no direct role in TGF-β signal transduction, as it lacks the signaling motif in the cytoplasmic domain [20]. It may function as a storage protein that regulates bioavailability of the ligand to target cells [21-23]. TβR-I and TβR-II are directly involved in TGF-β signaling, for these receptors contain serine/threonine kinases [24]. Each of the receptors possesses an extracellular region, a single transmemberane domain, and cytoplasmic signaling domain, which contain a serine/threonine kinase domain. Current understanding is that TβR-II binds TGF-β first and then recruits TβR-I. Signaling can only occur as a heteromeric complex [25]. Because of the knowledge that both TβR-I and TβR-II are required for TGF-β signaling, a loss of expression or functioning of either one of the receptors will lead to TGF-β insensitivity, which is common in cancer cells. In prostate cancer, it is well established that a loss of TGF-β receptors has been associated with high Gleason grade [26] and with a reduced survival period [27].

Role of Smad's in TGF-β Signaling

TGF-β and related factors use a simple mechanism to transmit signals. Binding of the ligand causes the assembly of a receptor complex that phosphorylates proteins of the Smad family, which consists of closely related proteins that bind DNA and recruit transcriptional co-activators or co-repressors. Phosphorylation causes Smad's to move into the nucleus, where they assemble complexes that control gene expression. Therefore, Smad's are a signal mediator and transfer signal from cytoplasmic into specific nucleus target genes [15, 28, 29].

Receptor-regulated Smad (R-Smad's) requires TGF-β-induced phosphorylation to assemble transcription regulatory complexes with partner Smad's (co-Smad's). R-Smad's can move into the nucleus by itself but, to be accessible to membrane receptors, R-Smad's are tethered in the cytoplasm by proteins such as SARA (Smad anchor for receptor activation). The type I receptor is kept inactive by a wedge-shaped GS region, which presses against the kinase domain, dislocating its catalytic center [28-30]. In the ligand-induced complex, the type II receptor phosphorylates the GS domain and activates the type I receptor, which catalyzes R-Smad phosphorylation. Phosphorylation decreases the affinity of R-Smad's for SARA and increases their affinity for co-Smad's. The resulting Smad complex is free to move into the nucleus and functions as transcriptional co-activators or co-repressors. Smad's can contact DNA, but effective binding to particular gene regulatory sites is enabled by specific DNA-binding cofactors. R-Smad's that move into the nucleus may return to the cytoplasm, but the ubiquitylation- and proteasome-dependent degradation in the nucleus provide way to terminate TGF-β responses [28,29,31]. Smad-2 and Smad-4 are frequently mutated in particular tumor subsets, suggesting that they may act as tumor suppressors. In addition, several oncogenic proteins can interact and inhibit the function of Smad proteins [8, 12, 28, 29, 32].

3. TGF-β IN NORMAL PHYSIOLOGY

Inhibition of cell proliferation is one of the TGF-β actions in epithelial, endothelial, hematopoietic, neural, and certain types of mesenchymal cells. Escape from this inhibition is a hallmark of many cancer cells. TGF-β is effective at inhibiting cell cycle progression during G1. In most cases this growth arrest effect is reversible, but in some cases, it is associated with cell apoptosis or cell death [28, 29]. Two classes of anti-proliferative gene responses are involved in TGF-β growth arrest: downregulation of c-*myc* and expression of inhibitors to cyclin-dependent kinases (cdks). The Myc family members (Myc, N-Myc, and L-Myc) is known to deregulate cell growth by promoting continuous, mitogen-independent, cell cycle progression [33-37]. The second class is cdk-inhibitors, which include the induction of p15 and p21 and downregulation of cdc 25A. Most cells that are growth inhibited by TGF-β have different combinations of cdk-inhibitory responses. C-Myc antagonizes TGF-β signaling by acting as a repressor of cdk-inhibitory responses. Downregulation of c-Myc is thus necessary for TGF-β-induced cell cycle arrest [28, 29, 38].

In addition to causing reversible cell cycle arrest in some cell types, TGF-β can induce programmed cell death in others. In fact, apoptosis induced by TGF-β family members is an essential component of the proper development of various tissues and organs [39, 40]. TGF-β induced apoptosis and the selective elimination of preneoplastic cells may also be involved in the tumor suppression mediated by TGF-β [41]. Just as loss of TGF-β mediated growth arrest might predispose a cell to cancer, loss of TGF-β mediated apoptosis may permit selective accumulation of premalignant cells [28, 29].

4. TGF-β IN TUMOR CELLS

Two important mutational events appear to be associated with malignant transformation. They are the loss of the sensitivity to the inhibitory effect of TGF-β and the acquisition of the ability to express an increased level of TGF-β. The following discussion expands this notion.

Down-regulation of TGF-β Sensitivity in Tumor Cells

Tumor cells often escape from TGF-β-induced growth arrest and apoptosis. **In head and neck tumors**, squamous cell carcinomas have decreased expression levels of TGF-β receptors [42]. Miss-sense mutations of TβR-II receptor have been noticed in two head and neck squamous carcinomas cell lines [43]. Tumor cells with less differentiation or with the least differentiation have a little or no expression of TβR-II receptor [44]. Despite frequent loss of heterozygosity (LOH) on chromosome 18q, only rare Smad-4 mutations have been reported suggesting that tumor suppressors other than Smad-4 are implicated in head and neck cancer progression [45, 46]. **In lung cancer,** reduced expression of TβR-II is seen frequently, especially in adenocarcinoma and in small cell lung carcinomas [26, 47, 48]. Several mutant forms of Smad-2 and Smad-4 have been described in lung cancer [49]. On the other hand, frame-shift mutations in TβR-II, IGFIIR, BAX, hMSH3, and hMSH6 are virtually absent in lung cancers [50]. **In breast cancer,** TβR-II downregulation is observed in breast cancer and appears to be due to a cellular trafficking defect whereby most TβR-II remains in the cytosol [51]. Smad-4 mutations have essentially not been reported in breast cancer. Only one breast cancer cell line, MDA-MB-468, harbors a homozygous deletion of the gene [52]. **In malignancies the digestive tract organ,** TβR-II dominant-negative mutation, that is not associated with MSI, has been reported in esophageal carcinoma [53]. Although MSI occurs in a third of esophageal carcinoma, TβR-II frame-shift mutations have not been reported [53, 54]. Haploid loss of Smad-4 has been shown to initiate gastric polyposis and cancer in mice [55, 56]. Mutations of TβR-II are found in more than 20% of colon cancers and TβR-II frame-shift mutations are present in 70-90% of colon cancer with microsatellite instability [57-59]. Furthermore, TβR-II mutation in sporadic colorectal polyps is a rare occurrence suggesting that TβR-II frame-shift mutations do not have a pathogenic role in early stages of colorectal cancer development [60, 61]. At the time when TβR-II mutations are detected, numerous other genes implicated in colon cancer, such as BAX, IGFRII, hMSH3, hMSH6, and TCF-4 are also mutated [59, 62]. Contrary to TβR-II frame-shift mutations associated with MSI that portend a good prognosis in colon cancer, Smad-4 mutations appear to be associated with aggressive disease [63]. In this disease, like in sporadic colorectal cancer, Smad-4 acts as a tumor suppressor gene [64]. TβR-I, TβR-II as well as Smad-4 mutations have been observed in Biliary cancer cell lines [65-67]. In pancreas cancer, the evidence corroborated that Smad-4 inactivation occurs late in the stage of histologically recognizable carcinoma [68]. Micro-satellite instability is a rare event in hepatic carcinogens, which is occasionally associated with TβR-II frame-shift mutations [69]. Smad-2 and Smad-4 somatic mutations occur in hepatic carcinoma [70]. **In genito-urinary cancers,** decrease expression of TβR-I is associated with a poor prognosis in patients with bladder transitional cell carcinoma [71] and with prostate cancer [27]. In a mouse renal cell carcinoma model, TβR-II expression is not detected in these cells [72, 73]. **In Gynecological malignancies,** TGF-β1 mRNA is down-regulated in endometrial carcinoma [74]. TβR-II mutations have been reported in cervical cancer [75]. TβR-II mutations are frequently in cell lines but rare in primary lesions [76]. Loss of TβR-II expression is common in certain studies and rare in others [77, 78]. Mutations of the Smad-4 promoter that have the potential to suppress or silence Smad-4 transcription have been reported in ovarian cancer [79]. **In hematological malignancies,** resistance of TGF-β1 correlates with a reduction of TβR-II expression in Burkitt's lymphoma and Epstein- Barr virus-transformed B lymphoblastoid cell lines [80]. Micro-satellite instability and frame-shift mutations in BAX and TβR-II genes are rarely seen in acute lymphoblastic leukemia *in vivo* but they are not uncommon in T-cell malignancies [81, 82]. **In skin cancer,** inactivation of TβR-II accelerates skin carcinogenesis at both earlier and later stages and increased angiogenesis is one of the important mechanisms of accelerated tumor growth and metastasis [83]. Decreased expression of TβR-I is associated with increased aggressiveness in human epithelial skin tumor [84].

Over-production of TGF-β by Tumor Cells

The association of TGF-β secretion with cancer is strongest in most advanced stages of tumor progression. The increased TGF-β secretion weakens the immune system and can exacerbate the malignant phenotype of tumor cells, contributing to tumor invasion and metastasis. There are ample examples that tumors over-produce TGF-β. **In brain tumor,** gliomas secrete TGF-β2 and thereby downregulate the expression of the adhesion molecule VCAM-1 on both the glioma cells and the cerebral microvessel endothelial cells [85]. Genomic instability occurs in glimas and TβR-II frame-shift mutations have occasionally been reported [86]. **In head and neck tumors,** patients with Epstein Barr associated nasopharyngeal carcinoma have elevated serum levels of TGF-β1 [87]. Patients with metastatic head and neck squamous cell carcinomas have elevated serum levels of TGF-β2 [88]. **In lung cancer,** patients frequently present with elevated serum levels of TGF-β1. Persistence of an elevated level after treatment is indicative of persistent disease [89]. **In breast cancer,** treatment of TGF-β1+/- heterozygous mice with tumor induction protocols results in a much higher number of malignant mammary tumors that in TGF-β1+/+ mice [90]. **In digestive tract organ malignancies,** high level of expression of TGF-β1 in the gastric mucosa of patients with a diagnosis of gastric cancer was recently reported. An interesting finding of the study was that the majority of the patients' first-degree relatives also expressed TGF-β1 in their gastric mucosa. In contrast, only one of 19 individuals without a family history of gastric cancer expressed TGF-β1 in the gastric mucosa. The induction of in the TGF-β1 expression in first degree relatives of patients with gastric cancer points to the presence of specific molecular alterations in a subgroup of individuals with an increased risk of developing gastric cancer [91]. In rat transformed colon cancer cells, TGF-β1 increase the synthesis of COX-2 mRNA and renders it more resistant to degradation [92]. A recent report suggests that insulin-like growth factor binding protein3 (IGFBP-3) mediates the TGF-β1-induced proliferation of metastatic colon carcinoma cell lines [93]. Biliary Cancer Cell Lines are resistant of TGF-β mediated growth inhibition [94]. In liver cancers, mRNA levels as well as immunostaining of TGF-β1, TGF-β2, TGF-β3 are markedly increase in liver cancer whereas TβR-I, and TβR-II immunostaining is essentially unchanged as compared with normal liver tissue [95].

5. TUMOR CELLS RESPONSES TO TGF-β

All epithelial and hematopoietic cell lines are highly sensitive to the inhibitory effect of TGF-β. In the normal cells, TGF-β acts as a tumor suppressor by inhibiting cell growth or by promoting cellular differentiation or apoptosis. Upon malignant transformation, virtually all cells become, at least partially, resistant to TGF-β. The resistant is due to inactivating mutations or less of expression of the genes for one or more know components of the TGF-β signaling pathway [58, 96, 97].

In contrast to the anticipated inhibitory effect of TGF-β, tumor progress in the face of TGF-β. This paradox warrants a closer look. In tumor cells, two major events occur regarding TGF-β action. They are the loss of expression of functional TGF-β receptors and overproduction of TGF-β. The loss of expression of functional TGF-β receptors provides a growth advantage to cancer cells over their benign counterparts. In addition to a loss of sensitivity to TGF-β, cancer cells are able to overexpress TGF-β, leading to more aggressive phenotypes [98, 99]. The overproduction of TGF-β by cancer cells has a multitude of adverse consequences. TGF-β can promote extracellular matrix production, induce angiogenesis, and inhibit host immune function. The biological consequence of these activities is an enhanced tumorigenicity [5, 99].

The overexpression of TGF-β from cancer cells alters the host-tumor interaction, which consequently facilitates tumor growth. TGF-β can inhibit the host immune system. This effect of TGF-β seems to play a significant role in facilitating MATLyLu rat prostate tumor growth in syngeneic hosts. MATLyLu cell line has a similar biochemical and histologic profile to that of late-stage human prostate cancer. It is either not immunogenic or only weakly immunogenic [100]. The classical studies by Steiner and Barrack (1992) and Barrack (1997) demonstrated that an overproduction of TGF-β1 in MATLyLu cells was growth-inhibitory *in vitro* but growth-stimulatory *in vivo*. Results of a recent study [101] showed that MATLyLu cells transfected with a TGF-β1 antisense expression vector reduced TGF-β1 production. These transfected cells proliferated *in vitro* at a much greater rate than that of wild-type MATLyLu cells. Yet, they either failed to form tumors or grew smaller tumors than did the wild-type cells.

6. HOST IMMUNITY IN THE PRESENCE OF TUMOR DERIVED TGF-β

Impaired Immune Function in the Presence of Tumor Derived TGF-β

A common property of cancer cells is that they over-produce TGF-β. Since TGF-β is an inhibitory growth factor, theoretically, it should be able to inhibit tumor growth. However, tumor cells have acquired the ability by becoming insensitive to TGF-β.

This is true for many cancers (see above section). Our early studies have shown that prostate cancer cases, especially with high Gleason grades, have lost statement of at least one or both TGF-β receptors [45]. Furthermore, the loss of TGF-β receptors has an impact on survival of prostate cancer patients [26]. This property provides a mechanism for cancer cells to escape the autocrine inhibition by TGF-β but, at the same time, high levels of TGF-β produced by these cells are highly immuno-suppressive.

Removal of TGF-β from Tumor Cells Rendering Them Immunogenic

In another way, theoretically, if one can remove TGF-β from the cancer cells, their growth should be inhibited by the host immune system. This was confirmed by our study [101]. MATLyLu rat prostate cancer cells are extremely aggressive and they produce high levels of TGF-β. When MATLyLu cells were genetically engineered to reduce the statement of TGF-β1, they fail to develop tumors in syngeneic hosts (Copenhagen rats) but tumor developed in immunodeficient hosts (nude rats). This is a proof-of-principle to demonstrate that TGF-β produced by tumor cells was a potent immunosuppressor.

7. TGF-β IN IMMUNE HOMEOSTASIS

TGF-β produced by tumor cells may diminish the effectiveness of antitumor T-cell immune responses [102-104]. The main function of TGF-β is to inhibit the growth and activities of T cells. It is viewed as an "anti-cytokine" because, in addition to its action on T cells, it can inhibit many functions of macrophages, B cells, neutrophils, and natural killer cells by counteracting the action of other activating factors. Although it is a "negative regulator" of the immune response, it stimulates wound healing the synthesis of collagen. TGF-β is produced by many kinds of cells, including T cells, B cells, and macrophages. The role of TGF-β is to suppress the immune response when it is no longer needed after an infection and to promote the healing process [105].

TGF-β is a Potent Immunosuppressant

TGF-β has been implicated in tumor-induced immunosuppression [106]. One of the reasons that an overstatement of TGF-β in cancer cells promotes tumor growth *in vivo* can be attributed to the fact that TGF-β is a potent immunosuppressant [107,108]. TGF-β is a powerful inhibitor of T and B cell proliferation. Therefore, an excess of TGF-β can inhibit host immune responses. This immunosuppressive role of TGF-β can be best demonstrated in TGF-β1 knockout mice. These animals are unable to survive beyond 21 days of age due to a severe widespread inflammatory reaction. These animals could survive longer, if they were treated with antibodies to MHC antigens or if they are rendered athymic [109].

Non-immune Host Cells in TGF-β Induced Immuno-suppression

Tumor-induced immunosuppression is a key mechanism by which tumors can evade host immunosurveillance. Aside from a direct inhibitory effect of TGF-β on immune cells, TGF-β can act on non-immune cells and can contribute to the immunosuppressive effect in the host. Interactions between thymic stromal cells and immune cells are the basis for T cell selection and have an impact on final T cell repertoire [110]. For example, TGF-β expressed from thymic stromal cells regulates the differentiation of CD4+CD8+ double positive stages [111]. Another example is the inhibitory effect of TGF-β on the production of IL-7 by non-lymphoid stromal cells, which are important for the development of B cells [112].

TGF-β in Tumor Immunology

TGF-β is the principal immunosuppressive component derived from tumor cells [106,113]. Modification of highly immunogenic C3H tumors with a TGF-β expression vector allowed for growth and escape from immunosurveillance *in vivo* despite an apparent lack of downregulation of MHC class I or tumor-specific antigen [114]. Neutralization of TGF-β resulted in abrogation of MCF-7 tumors [115]. A complete eradication of rat glioma tumors was noted when an antisense TGF-β construct was introduced into tumor cells ex vivo and then locally reintroduced into the tumor-bearing host [116]. A similar approach was reported with the Dunning rat prostate tumor MATLyLu [101]. In a mouse thymoma model, tumor cells engineered to secrete a soluble TβR-II resulted in a suppression of tumorigenicity [117]. These reports support the notion that TGF-β production by tumor cells inhibits immunosurveillance and that elimination of TGF-β from tumor cells enhances host immune response.

8. TGF-β BASED IMMUNOTHERAPY FOR CANCER.

TGF-β Insensitivity in Autoimmune-like Disease

For the sake of simplicity, we divide the etiology of autoimmune-like disease into thymic origin and/or peripheral tissue origin [111, 118], 1991. Many reports implicate TGF-β in the pathogenesis of autoimmune-like disease. Systemic administration of TGF-β suppressed the symptoms of experimental encephalomyelitis (EAE) while antibodies to TGF-β enhanced the disease [119,120]. Mice null for TGF-β1 developed autoimmune-like syndrome including enhanced expression of MHC class I and II antigens, circulating SLE-like IgG antibodies to nuclear antigens, pathogenic glomerular IgG deposits, and progressive infiltration of lymphocytes into multiple organs [121,122]. Development of autoimmunity is normally resulted in selection processes in the thymus or through mechanisms that maintain tolerance in peripheral tissues. In the thymus, negative selection takes place at the CD4+CD8+ double positive stage [123]. Since TGF-β regulates the maturation of these double positive cells, it is likely that, in the absence of TGF-β action, double positive cells are generated rapidly for their appropriate elimination [111,124]. Mechanisms of maintenance of peripheral tolerance can include a balance of reactive and suppressor (or regulatory) T cells [125]. TGF-β, again, plays a major role in this process [110,126,127]. However, mechanisms of TGF-β insensitive immune cells in autoimmunity remain undefined.

Transplant of TGF-β Insensitive Bone Marrow Leads to Myeloid Expansion and Elimination of Tumor Cells

In this section, we describe the development of a murine model of TGF-β insensitivity limited to the hematopoietic tissue of adult C57BL/6 mice. Unlike the lymphoproliferative syndrome observed in TGF-β1 deficient mice, the disruption of TGF-β signaling in bone marrow derived cells leads to dramatic expansion of myeloid cells, primarily monocytes/macrophages, and is associated with cachexia. Surprisingly, there was a notable absence of T cell expansion in affected animals, despite the observed differentiation of most cells in the T cell compartment to a memory phenotype [128]. When tumor cells (mouse B16 melanoma cells or mouse TRAMP prostate cancer cells) were injected into these animals, tumor cells were eliminated [129].

Adapting a TGF-β Based Protection Strategy to Enhance Anti-tumor Immunity

Another example of TGF-β insensitivity in immune cells resulted in tumor elimination was the model of Epstein- Barr virus (EBV)-specific CTLs, which were transduced with a retrovirus vector expressing the dominant-negative TGF-β type II receptor HATGF-βRII Δcyt for the treatment of EBV-positive Hodgkin disease. HATGF-βRII Δcyt transducted CTLs were resistant to the antiproliferative and anticytotoxic effects of exogenous TGF-β. Additionally, these transduced CTLs continued to secrete cytokines in response to antigenic stimulation. Long-term expression of HATGF-βRII Δcyt did not affect CTL function, phenotype, or growth characteristics. Tumor-specific CTLs expressing HATGF-βRII Δcyt should have a selective functional and survival advantage over unmodified CTLs in the presence of TGF-β secreting tumors [130].

CONCLUSION

Based on the above discussion, it is encouraging that there is a possibility for us to achieve a cure in cancer using TGF-β insensitive immune cells in gene therapy. The host immune system offers a natural defense program against cancer. But, this natural immunosurveillance is rendered ineffective by an overproduction of TGF-β derived from the tumor cells. In the past, many attempts have been made in an effort to boost the host immune system with the intention of a cure for cancer. Unfortunately, these efforts were met with little success, possibly due to a lack of consideration of the powerful role of the tumor-derived TGF-β in immunosuppression. The present discussion illustrates that TGF-β signaling plays a key role in regulating our immune system. Rendering the host immune cells insensitive to TGF-β in a gene therapy program offers a hope for us to successfully combat against cancer. Therefore, it is important that our future research should focus on the development of a TGF-β based therapeutic strategy for the treatment of cancer.

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